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Expression and purification of active human internal His_6-tagged L-glutamine: D-Fructose-6P amidotransferase I

Céline Richez, Joachim Boetzel, Nicolas Floquet, Kinnera Koteshwar, Julie Stevens, Bernard Badet, Marie-Ange Badet-Denisot *

Institut de Chimie des Substances Naturelles, ICSN-CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

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Abstract

Human L-glutamine: D-fructose-6-phosphate amidotransferase (Gfat1), a recognized target in type 2 diabetes complications, was expressed in Sf9 insect cells with an internal His_6-tag and purified to homogenity. Two different microplate assays that quantify, respectively D-glucosamine-6-phosphate and L-glutamate were used to analyze the enzyme kinetic properties. The recombinant human L-glutamine: D-fructose-6-phosphate amidotransferase isoform 1 exhibits Michaelis parameters $K_m^{Fru\text{-}6P} = 0.98$ mM and $K_m^{Gln} = 0.84$ mM which are similar to the values reported for the same enzyme from different sources. The stimulation of hydrolysis of the alternate substrate L-glutamine para-nitroanilide by D-fructose-6P (Fru-6P) afforded a K_d of 5 μM for Fru-6P.

Keywords: Human L-glutamine: D-fructose-6-phosphate amidotransferase; Internal His_6-tag; Hexosamine pathway; NtN hydrolase; Type 2 diabetes

The enzyme L-glutamine: D-fructose-6P amidotransferase, EC 2.6.1.16, (GlmS, Gfa or Gfat according to the enzyme source) catalyzes the first and rate limiting step [1] of the *de novo* biosynthesis of hexosamines (Scheme 1), leading to the formation of uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc).[1]

UDP-GlcNAc is an activated precursor of numerous aminosugar containing macromolecules, including chitin and mannoproteins in fungi, peptidoglycans and lipopolysaccharides in bacteria and glycoproteins in mammals. Human Gfat1 has been convincingly shown to participate in the vascular complications of type II diabetes [2] and as such is an interesting target in medicinal chemistry.

The recombinant bacterial form, GlmS, has been extensively studied [3] together with other members of the N-terminal nucleophile (NtN) hydrolase class [4–7]. Each enzyme subunit is organized into two domains, the glutaminase domain responsible for hydrolysis of L-glutamine (Gln) into L-glutamate (Glu) and the isomerase domain responsible for ketose/aldose isomerization of the RR′C=NH aminosugar phosphate adduct. These two domains are connected by an intramolecular channel [8] responsible for the transfer of ammonia.

Forty years separated the first partial purification of Gfat from rat liver [9] from the first eukaryotic protein characterization from either *Candida albicans* or rat liver [10–12]. Human Gfat1 was first expressed as a fusion

* Corresponding author. Fax: +33 1 69077247.
E-mail address: badetma@icsn.cnrs-gif.fr (M.-A. Badet-Denisot).

[1] *Abbreviations used:* APAD, 3-Acetylpyridine adenine dinucleotide; DTT, Dithiothreitol; EDTA, ethylenediaminetetra acetic acid; Fru-6P, D-fructose-6P; GlcN-6P, D-glucosamine-6P; GDH, L-glutamate dehydrogenase; Gln, L-glutamine; GSH, glutathion; GST, glutathione S-transferase; Glu, L-glutamate; His_6-225-GlmS, GlmS with six His in position 225; His_6-240-GlmS, GlmS with six His in position 240; HSP, Hexosamine synthetic pathway; moi, multiplicity of infection; NAD, Nicotinamide Adenine Dinucleotide; pfu, plaque-forming units; rhGfat1, recombinant human L-glutamine: D-fructose-6-phosphate amidotransferase isoform 1 containing six His in position 298; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine; WT, wild-type.

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L-Glutamine + D-Fructose-6P ⟶ L-Glutamate + D-Glucosamine-6P

Scheme 1.

protein with glutathione S-transferase (GST) in BSC40 cells using a viral expression system. This construction afforded a protein of low specific activity [13]. Using an enzyme overexpressed in low yield in insect cells, Broschat et al. reported detailed investigation of its catalytic properties and inhibition by D-glucosamine-6P (GlcN-6P) and UDP-GlcNAc using a very sensitive radiometric assay [14,15]. Our initial difficulties associated with human Gfat1 expression and manipulation prompted us to introduce a tag in the protein sequence.

Sequence alignments indicate that the human enzyme (hGfat) differs from its bacterial counterpart (GlmS) at the level of the C-terminus of the Gln binding site (i.e. Gly185), whereas the composition of the active site region is essentially the same. From the 3D structure of GlmS, four reasonable positions favorable to a His_6-tag insertion were selected.

The corresponding mutants were expressed and characterized allowing identification of a single position 225 which tolerated a His_6-tag insertion. The optimized construction was then extrapolated to human Gfat1 and the corresponding protein was expressed in insect cells. The His_6-298 recombinant human enzyme (rhGfat1) purified in two steps possesses a specific activity almost 10-fold higher than that previously reported by Broshat [15]. From gel filtration and sedimentation equilibrium analysis the enzyme appears as a homotetramer.

For clarity, GlmS refers to bacterial D-glucosamine-6P synthase and rhGfat1 refers to recombinant human D-glucosamine-6P synthase isoform 1 throughout the text.

Materials and methods

Materials

The pFastBac1 donnor vector, competent DH10Bac *Escherichia coli* cells were purchased from Invitrogen; competent HB101 *E. coli* cells and competent DH5α *E. coli* were from Promega.

Antibiotics, Bluo-gal, IPTG, D-fructose-6P (Fru-6P) and other reagents were from Sigma; EDTA-free cocktail inhibitor and Pefabloc were from Roche Applied Science; TCEP was from Fluka.

The pMA1 plasmid which harbors the *glmS* gene encoding *E. coli* GlcN-6P synthase was previously described [16].

Restriction and modification enzymes were purchased from New England Biolabs. Platinum pfx polymerase was from Roche Applied Science. Purification of His_6-tagged proteins was performed on a 5 mL Histrap FF crude column using an AKTA EXPLORER 10S system (GE Healthcare). DNA molecular weight markers were from Bio-Rad and protein molecular weight markers were from Qiagen and GE Healthcare.

Buffer A: 50 mM KCl, 50 mM KPO_4, 1 mM DTT, 2 mM Fru-6P and Roche EDTA-free cocktail inhibitor, pH 7.5.

Buffer B: 300 mM NaCl, 50 mM KPO_4, 5% glycerol, 30 mM imidazole, 1 mM TCEP, 1 mM Fru-6P,1 mM PMSF, Roche EDTA-free cocktail inhibitor, pH 7.5.

Buffer C: 300 mM NaCl, 50 mM KPO_4, 10% glycerol, 1 mM TCEP, 1 mM Fru-6P, 1 mM PMSF, pH 7.5.

Buffer D: 50 mM NaCl, 150 mM KPO_4, 10% glycerol, 1 mM TCEP, 1 mM Fru-6P, 1 mM EDTA, pH 7.5.

Buffer A′: 300 mM NaCl, 50 mM KPO_4, 5% glycerol, 30 mM imidazole, 5 mM TCEP, 5 mM Fru-6P, 0.4 mM Roche Pefabloc SC, 2 μM phosphoramidon, Roche EDTA-free cocktail inhibitor, pH 7.7.

Buffer B′: 300 mM NaCl, 50 mM KPO_4, 10% glycerol, 5 mM TCEP, 5 mM Fru-6P, 0.4 mM Roche Pefabloc SC, 2 μM phosphoramidon, pH 7.7.

Buffer C′: 50 mM NaCl, 150 mM KPO_4, 10% glycerol, 5 mM TCEP, 5 mM Fru-6P, 2 mM EDTA, pH 7.7.

DNA manipulations

Isolation of plasmid DNA was carried out according to the Qiagen plasmid kit protocol. DNA fragments were isolated from agarose gels following the Geneclean II DNA extraction kit protocol (Bio 101, Inc., Vista, CA, USA). DNA digestions were carried out according to enzyme manufacturer's instructions. DNA fragments were ligated and *E. coli* cells were transformed according to standard methods [17].

Construction of bacterial expression plasmids

pGEX-GST-N-terminal GlmS: this recombinant plasmid containing a N-terminal GST fusion of *E. coli* D-GlcN-6P synthase gene was kindly provided by Dr. J.E. Kudlow.

pMA1-Go was obtained as previously described [18]. pMA1-225-His_6 , pMA1-240-His_6 and pMA1-C-terminal-His_6 were obtained following a similar protocol. Cloning of the His_6-tagged enzyme was performed by PCR from the pUC 118 vector containing the *E. coli glms* gene (pMA_1) as a template. The primers used for the pMA1-225His_6 containing the His_6-tag after threonine residue 225 were (His_6-tag sequence underlined): Start-*Bsi*WI 5′-GCG TAC GGT ACA GTG ATC ATG GAC TCC CGT C-3′ and End-RV 5′-CGA TAT CCT GAC GTT

TTA CTT CCG CGC CGT GAT GGT GAT GGT GAT GAG TTT TAT CGA AG-3′. The primers used for pMA1-240-His_6 containing the His_6-tag after threonine residue 240 were: Start-RV 5′-CAG GAT ATC GAA TCC AAT CTG CAA TAT CAT CAC CAT CAC CAT CAC GAC GCG GGC G-3′ and End-*Nhe* 5′-ACC TGC TAG CGA TTC AAA CCA GTA GCG GG-3′. The primers used for pMA1-CtHis_6 containing the His_6-tag in the C-terminal position were: Start-*Bss*HII 5′-GTT CGC GCG CGT GGC CGT GGC GGT CAG TTG TAT GTC-3′ and End-*Bam*HI 5′-GCC CCG CTG GAT CCG GGC ATC CAT TTA TTA GTG ATG GTG ATG GTG ATG CTC AAC CGT AAC CG-3′. The purified PCR products were digested and inserted between *Bsi*WI and *Eco*RV sites or *Eco*RV and *Nhe*I sites or *Bss*HII and *Bam*HI sites, replacing its homolog in pMA1, giving, respectively, the three recombinant constructs mentioned above. The identity of the recombinant plasmids was confirmed by restriction analysis and DNA sequencing.

Expression and purification of different tagged-GlmS

E. coli BL21 culture (250 mL) harboring pGEX-GST-Nt*glms* expression plasmid in LB medium supplemented with ampicillin (100 μg/mL) was grown for 4 h at 30 °C followed by 1 mM IPTG induction for 3.5 h at 30 °C. After centrifugation, the pellet was resuspended in the starting buffer A and disrupted by sonication yielding 2.5 mL of crude extract (12 mg/mL) which was purified on a glutathion-Sepharose column (2 mL). The fusion protein was detected by GST activity before cleavage with thrombin as described by Habig et al. [19] to afford *E. coli* GlmS (67 kDa) and GST (26 kDa) as visualized from SDS–PAGE analysis.

E. coli HB101 cells were transformed with pMA1-Ct His_6, pMA1-240 His_6 or pMA1-225 His_6 expression plasmid, respectively. One liter of LB medium supplemented with ampicillin (100 μg/mL) was grown overnight at 37 °C, without induction by IPTG. The purifications of these three recombinant proteins were conducted similarly. The pellet (7.1 g) resuspended in buffer B (50 mL) was sonicated. The lysate was submitted to ultracentrifugation (110,000*g*, 35 min, 4 °C) before loading at 1 mL/min on a 5 mL HisTrap FF crude column (GE Healthcare) equilibrated in buffer C. After washing with buffer C containing 50 mM imidazole (10 column volumes), elution was carried out at 1.5 mL/min stepwise with imidazole concentrations 0.1, 0.3, 0.5 and 1 M in buffer C (8–10 column volumes).

The concentrated active fractions were then loaded on Superose 12 10/30 (GE Healthcare) equilibrated in buffer D (0.4 mL/min).

Production of recombinant baculovirus

The *Eco*RI fragment of cDNA corresponding to *gfat* gene cloned at the *Eco*RI restriction site of pCRII vector was a gift from Dr. D.A. McClain. An internal His_6-tag residue was introduced in position 894 [20] by polymerase chain reaction with Platinum pfx polymerase and appropriate primer pairs: Start-*Aat*II-$His_6$5′-TGG ACG TCT TTC TAT CCA TCG AAT TAA ACG AAC TGC AGG ACA TCA CCA TCA CCA TCA CGA TCA CCC CGG ACG-3′ and End-*Hinc*II 5′-CAA AGT TGA CTC TTC CTC TCA TTG TGT TCA CGA CAG ACT CTG GC-3′. After digestion by *Aat*II and *Hinc*II and purification, the amplicon (170 bp) was inserted at the corresponding restriction sites in the pCR II-*gfat* construction by an overnight ligation reaction at 16 °C. The *Xba*I–*Hin*dIII fragment of the recombinant pCRII-gfat-His_6 was cloned into the baculovirus donor plasmid pFastBac1 at the corresponding sites and verified by multiple restriction enzyme digestions (*Sma*I, *Acc*I/*Dra*I, *Pst*EI/ *Xba*I) and by sequencing. In order to remove two ORFs, the sequence before the ATG was mutated by PCR with the primer pair: Start-*Xba*I 5′-AAT CTA GAT TCA TGC TCG AGC GGC CGC CAG TGT GAT TGA TAT C-3′ End-*Afe*I 5′-ATT TTT ATC AGA GCG CTG GGG GTG GCT ATT GAC AGG-3′. The purified PCR fragment was digested by *Xba*I and *Afe*I. The new fragment replaced its homolog in pFastBac-gfat-His_6 and the new recombinant donor plasmid named pMAD1 was used for the transposition step in DH10Bac *E. coli*. Recombinant bacmid DNA was isolated from DH10Bac cells, verified by PCR and used to transfect Sf9 cells as according to manufacturer's instructions. The resultant baculovirus was verified by PCR and a viral stock of 50 mL was titrated by plaque assay (9.10^7 plaque-forming units (pfu)/mL).

Optimization of expression of recombinant human protein

Sf9 insect cells were grown at 28 °C in SF900II medium (50 mL, Life Technologies Ltd.) in shaker flasks at 100 rpm. Cells (2×10^6 cells/L) were infected with recombinant baculovirus at different multiplicities of infection (moi) of 0.1, 0.5 and 2 pfu/cell. Cells were harvested 24, 48, 72 and 96 h post-infection. Two milliliter aliquots of each of the latter were centrifuged (2500*g*, 3 min at 4 °C) and each cell pellet were washed in 20 mM Tris–HCl, pH 7, centrifuged (4000*g*, 15 min at 4 °C) and assayed for mini purification in batches using a chelating-Sepharose FF charged in Ni^{2+}. The kinetics of production were analyzed by 10% SDS–PAGE gel stained with Coomassie blue and the western blot was revealed by anti-penta-His antibodies (Qiagen).

Expression and purification of recombinant Gfat1 protein

Sf9 insect cells were grown at 28 °C in SF900II medium (Life Technologies Ltd) in shaker flasks (10×1 L) at 100 rpm. When the density reached 2×10^6 cells/L, cells were infected with recombinant baculovirus at moi of 0.1 pfu/cell. Cells were harvested 72 h after infection, centrifuged (500*g*, 10 min at 4 °C) and washed in SF900II before storage at −80 °C.

The cell pellet (18.6 g) was suspended in 35 mL buffer A′ and disrupted at 4 °C using DynoMill (4500 rpm, 4 cycles 30 s, 40 g beads of 0.2 mm diameter). The crude extract was centrifuged (10,000*g* for 20 min at 4 °C) and the supernatant was cleared by ultra-centrifugation at 1,10,000*g* for 35 min at 4 °C. Purification was carried out by affinity chromatography over a 5 mL Histrap FF crude column on an AKTA EXPLORER 10S system (GE Healthcare). The lysate was loaded at 1 mL/min onto the column equilibrated in buffer B′ and unbound material was removed by washing at 3 mL/min with 10 column volumes of buffer B′containing 50 mM imidazole. The elution of the protein was carried out at 1.5 mL/min stepwise in buffer B′ with imidazole concentrations of 0.1, 0.3, 0.5 and 1 M (8–10 column volumes). Fractions containing rhGfat1 were pooled, concentrated by ultrafiltration (using Millipore concentrators, 50 kDa cut-off limit, 4 °C, 4000 g, 20 min) and aliquoted for storage at −80 °C for several months.

The protein concentration was estimated by the method of Bradford using bovine serum albumin (BSA) as the standard (0–12 μg) [21].

Protein samples were analyzed by SDS–PAGE using 7.5% minigels (Mini-Protean III, Bio-Rad) [22]. Gels were stained with Coomassie R-250 blue stain and protein purity was determined by densitometry gel analysis.

Oligomerization degree determination

The degree of oligomerization of the rhGfat1 was determined by gel filtration and by analytical ultracentrifugation.

Gel filtration was performed on Superose 6 10/30 (GE Healthcare) equilibrated in buffer C′ at 0.35 mL/min. The column was calibrated by different markers (dextran blue M_r 2,000,000, β-amylase M_r 200,000, alcohol dehydrogenase M_r 150,000, BSA M_r 66,000, carbonic anhydrase M_r 29,000, acetone). The molecular weight of the native enzyme was obtained by interpolation of a plot of log molecular mass versus K_{av}.

Analytical ultracentrifugation was performed at the Pasteur Institute (PFBMI department) with a sample of rhGfat1 (3 mg/mL) in 50 mM KPO_4, 150 mM NaCl, 1 mM TCEP, 1 mM Fru-6P, 1 mM EDTA pH 7.5 at 4 °C (8,000 rpm, 48 h, Beckman XL-A). The experiment was followed by UV detection at 280 nm.

L-glutamate determination (glutaminase activity)

The L-glutamate dehydrogenase (GDH) coupled spectrophotometric assay was described in 1987 by Badet et al. [23]. The formation of Glu was also quantified by GDH coupled fluorometric assay using a Perkin Elmer Wallac Victor instrument. An excitation filter of 370 ± 14 nm and an emission filter of 460 ± 25 nm were used to follow the formation of APADH. The enzymatic mixture consisted of 100 mM KPO_4, 50 mM KCl, 1 mM EDTA, 1 mM APAD pH 7.5, with various concentrations of Fru-6P and Gln, and 2.7 U of GDH. The mixture was incubated at 37 °C in 96-well fluorescence plates with fluorescent readings every 0.7 s.

D-GlcN-6P quantification (synthase activity)

A modified Morgan–Elson procedure was used during purification of the enzyme [24]. One unit of specific activity was defined as the amount of the enzyme that catalyzed formation of 1 μmol GlcN-6P min^{-1} mg $protein^{-1}$. Kinetic parameters were determined from the production of GlcN-6P using the microplate Morgan–Elson assay [25].

Activation of glutaminase activity by Fru-6P

The activation of glutaminase activity by Fru-6P was determined by L-glutamic acid *p*-nitroanilide (GLUPA) hydrolysis [26] in 200 μL mixture (100 mM KPO_4, 50 mM KCl, 1 mM EDTA pH 7.5) and using 3 μg rhGfat1. The assay was initiated by the addition of GLUPA at a final concentration of 3 mM in rhGfat1 assay in presence of various concentrations of Fru-6P between 0.01 and 0.8 mM. Rate measurements were calculated from the slope of absorbance versus time using the kinetic reading mode of Molecular Devices SpectraMaxPlus plate reader at 405 nm. Typical assay duration was 15 min at 37 °C with absorbance readings every 10 s.

Determination of isoelectric point

The protein standards from Invitrogen (50 μg) and sample (9 μg in 20 mM lysine, 20 mM arginine, 15% glycerol) were loaded on Invitrogen gel pH 3–10 and run according to the manufacturer's instructions. After migration the gel was fixed by 30 min immersion in 12% TCA and 3.5% sulfosalicylic acid and revealed with Coomassie blue.

pH activity profile

0.8 μg of rhGfat1 was assayed for activity using the microplate Morgan–Elson test (see above) with appropriate concentrations of NaCl and 100 mM piperazine pH 5–5.5; MES pH 6–6.5; Na_2HPO_4 pH 7–7.5; Hepes pH 8–8.5; Tris pH 9; CAPS pH 9.5–10. The standards (0–40 nmol GlcN-6P) were used for each assay.

Circular dichroism

Circular dichroism (CD) spectra of the three enzymes (WTGlms, His_6-225-Glms and rhGfat1) were recorded at physiological temperature on a Jasco-J810 spectropolarimeter. A cell with 1 mm optical path length was used. Five scans were performed for noise reduction in the range 195–250 nm taking points every 1 nm, with a 50 nm min^{-1} scan rate, an integration time of 2 s and a 1 nm bandwidth. The protein concentrations were 0.15 mg/mL in a buffer of 20 mM KPO_4 buffer, 20 mM NaCl, 2 mM Fru-6P, 2 mM

TCEP, 2 mM EDTA and 1% glycerol, pH 7.5. The data are expressed as the per residue molar absorption in mdeg M^{-1} cm^{-1}. The deconvolutions of the CD spectra were performed with the CDPRO suite of programs [27] available at http://lamar.colostate.edu/~sreeram/CDPro. The results obtained were compared using three different methods: CONTINLL, SELCON3 and CDSSTR [28].

Results

Cloning, expression and purification of the variants of the bacterial enzyme

The expression plasmid pGEX-GST-Nt GlmS contains *glmS* gene fused with GST tag at the N-terminus. The resulting recombinant protein has a residual 0.1% synthase activity whereas the protein after cleavage with thrombin exhibited a specific activity of 0.7% of that of the wild type enzyme (Table 1).

Three overexpression plasmids containing DNA sequences encoding His_6-tagged GlmS were constructed. One of them encoding fusion of GlmS with C-terminus bound His_6-tag (His_6-Ct-GlmS) was obtained by exchange of the *Bam*HI/*Bss*HII fragment containing the His_6-tag into the *Bam*HI/*Bss*HII site of the pMA1. The two others coded for GlmS with internal His_6-tag at position 240 or 225 (GlmS amino acid numbering). Position 240 which belongs to the hinge region of the protein corresponds to the exposed site during controlled proteolysis by chymotrypsin [29]. The exposure of position 225 to the solvent was deduced from the observation of the 3D structure of the native protein [8]. These two expression plasmids were obtained by exchange of the His_6-tag containing fragment *Eco*RV–*Nhe*I and *Bsi*WI–*Eco*RV of pMA1, respectively. The absence of mutation within the gene was verified by sequencing.

Recombinant proteins were expressed in HB101 *E. coli* cells at about 25–35 mg/L of the culture. The activity of the affinity purified enzymes, summarized in Table 1, shows that both His_6-Ct GlmS and His_6-240-GlmS were strongly affected in their catalytic efficiency which was, respectively, 2.6% and 1% of that of the wild type whereas His_6-225-GlmS remained essentially as active as the WT.

The purification of His_6-225-GlmS from 1 L of culture (7.1 g pellet) afforded 36 mg of 99% pure enzyme (according to gel densitometry analysis) (Fig. 1). Despite an isoelectric point slightly higher than the wild type (6 *vs* 5.4) resulting from the addition of the basic residues from His_6-tag and a higher sensitivity to freeze-thaw cycles, His_6-225-GlmS remained stable at 4 °C for more than a week. With kinetic parameters very similar to WTGlmS (Table 3), it can be concluded that introduction of His_6-tag at position 225 of GlmS did not affect enzyme activity. CD analysis (see below) confirms the similar conformations of WT and His_6-225-GlmS (Fig. 2).

Table 1
Synthase Activity of wild type GlmS and of its affinity purified fusion mutants

Enzyme	Synthase activity (U/mg)
WTGlmS	8 ± 0.3
GST-Nt-GlmS	0.007 ± 0.001
His6-Ct-GlmS	0.21 ± 0.1
His6-240-GlmS	0.08 ± 0.02
His6-225-GlmS	8 ± 0.4

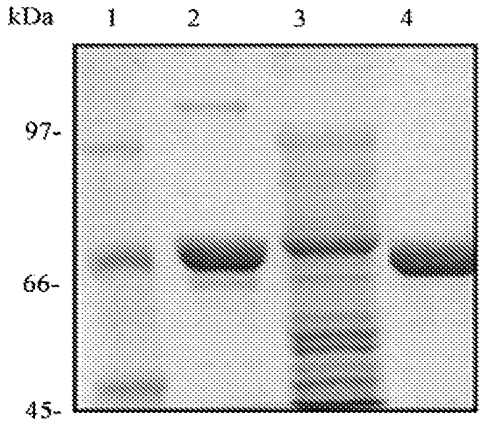

Fig. 1. SDS–PAGE Electrophoresis of His_6-225-GlmS purified from *E. coli* HB101. Proteins from each purification step were separated on 7.5% SDS–PAGE gel and stained with Coomassie blue. Lanes 1, the following molecular weight markers were used: phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumine (45 kDa); 2, 5 μg bacterial enzyme control; 3, 10 μg lysate extract; 4, 3 μg protein from affinity step.

Circular dichroism

The CD spectra at 37 °C of the three native enzymes WTGlmS, His_6-225-GlmS, rhGfat1 are shown in Fig. 2. The three curves presented a broad minimum around 210 and 220 nm characteristic of the presence of α-helical segments. Analysis of the spectra performed with CDPRO led to very similar results for the three proteins (24 ± 1% α-helix; 25 ± 1% β-sheet and 50 ± 0% coil) arguing for similar folding. These values were in good agreement with those extracted from the most recent X-ray structure of the bacterial enzyme [30] using the DSSP algorithm [31]: (27% α-helix; 20.5% β-sheet and 52.5% coil).

Cloning, expression and purification of human His_6-tagged Gfat1 from insect cells (rhGfat1)

An internal His_6-tag residue was introduced by PCR at position 894 in the nucleotide sequence of Gfat1 and the *Xba*I–*Hind*III fragment was cloned into the baculovirus donor plasmid pFastBac1 at the corresponding sites. The recombinant plasmid pMAD1 was used in the transposition step in DH10Bac *E. coli* to generate recombinant bacmid. After culture of Sf9 cells under optimized conditions (moi 0.1, 72 h post infection), the cells from 1 L of culture

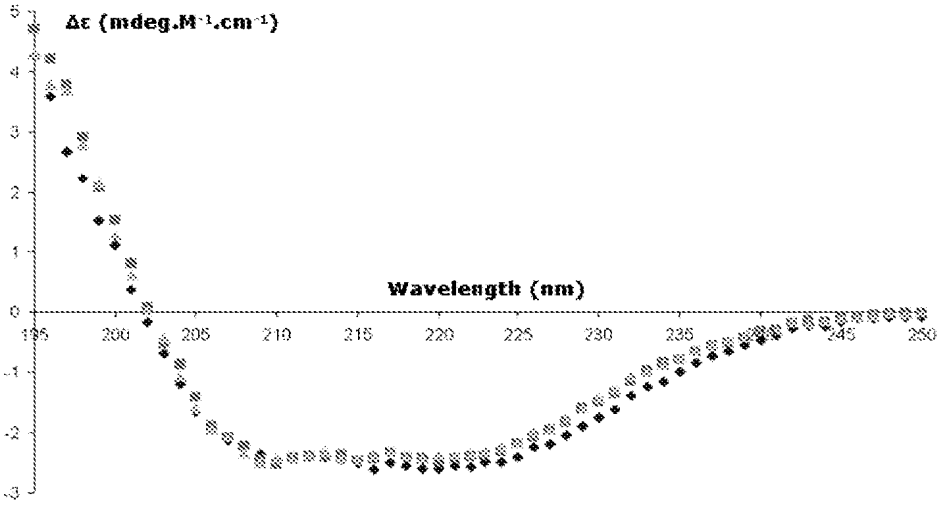

Fig. 2. Circular dichroïsm spectra at 37 °C of the three GlcN-6P synthases (0.15 mg/mL: rhGfat1 (blue rhomb), WTGlmS (orange triangle), GlmS-225 His_6 (pink square).

were harvested to afford 18.6 g of cell pellet. Among the different cell disruption techniques tested (French Press, sonication and Dyno-Mill), Dyno-Mill gave the best recovery of enzyme activity likely resulting from a better control of temperature at 4 °C. The addition of 5 mM TCEP and 5 mM Fru-6 P in the lysate buffer prevented the loss of enzyme activity, classically occurring upon cell breakage (Table 2). Stepwise elution with 0.1 M imidazole allowed removal of most of the contaminating histidine-rich insect proteins before elution with 0.3 M imidazole affording in one step a 85% pure protein as deduced from densitometry gel analysis (Fig. 3). The fractions containing the protein were desalted to avoid interference of imidazole with the enzymatic test, leading to a specific activity of 2.3 U/mg. After gel filtration on Superdex 200 10/300, the specific activity of the 95% pure protein reached 3.7 U/mg (Fig. 3). The purification of rhGfat1 from 1 L of insect cell culture (18.6 g frozen pellet) gave close to 5 mg of enzyme. The protein was stable at 4 °C in this buffer during several days.

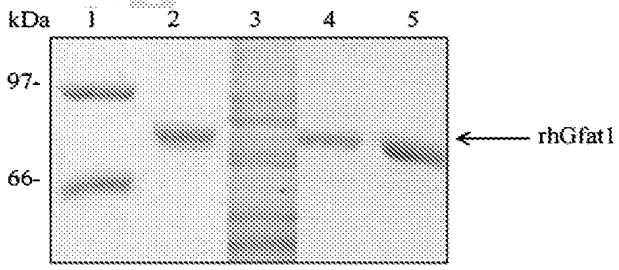

Fig. 3. SDS–polyacrylamide gel electrophoresis of rhGfat1 purified from insect cells. Proteins from each purification step were separated on 7.5% SDS–PAGE gel and stained with Coomassie blue. Lanes 1, molecular weight markers; 2, 2 μg rhGfat control; 3, 14 μg lysate extract; 4, 2 μg rhGfat1 from affinity step; 5, 2 μg rhGfat1 from gel filtration.

Characterization of rhGfat1

The equilibrium ultracentrifugation analysis data were submitted to curve fitting adjustment using ideal I program which showed that the enzyme exists only under one oligomerization state, a homotetramer of 306 ± 4 kDa, corresponding to 77 kDa per monomer. The molecular mass of rhGfat1 determined by size-exclusion chromatography on Superdex 200 and Superose 6 afforded from the log molecular mass versus K_{av} plot (data not shown) a value of 316 ± 3 kDa corresponding to 78 kDa per monomer, in good agreement with equilibrium ultracentrifugation results. The band observed on SDS–PAGE was close to 77 kDa consistent with results of previous methods (Fig. 3). The recombinant human enzyme exhibited an

Table 2
Purification of rhGfat1 overexpressed in insect cells

Purification step	Volume (mL)	Total protein[a] (mg)	Total activity[b] (U)	Specific actvity[c] (U/mg)	Yield (%)	Purification factor
Crude extract	41	660	39.6	0.06	100	1
Nickel column	30	10	23.0	2.3	58	38.3
Superdex 200	53	4.7	17.4	3.7	44	61.6

[a] Protein concentrations were determined by the method of Bradford using bovine serum albumin as standards.
[b] Enzyme units activity are defined as the amount (μmol) of GlcN-6P synthesized per min at 37 °C.
[c] Specific activity was expressed as units per mg of protein.

Table 3
Kinetic parameters of the three enzymes (WTGlmS, His_6-225-GlmS, rhGfat1)

Synthase activity	WTGlmS	His6-225-GlmS	rhGfat1
V_{max} (U/mg)	10.52 ± 0.55	7.87 ± 0.88	3.66 ± 0.37
K_m^{F6P}(mM)	0.99 ± 0.07	1.53 ± 0.21	0.98 ± 0.17
K_{cat} (S^{-1})	23.4	17.7	18.9

One unit of enzyme activity was defined as the amount required to catalyse the synthesis of 1 μmol of GlcN-6P per min for synthase activity and 1 μmol of Glu per min for hemisynthase activity at 37 °C.

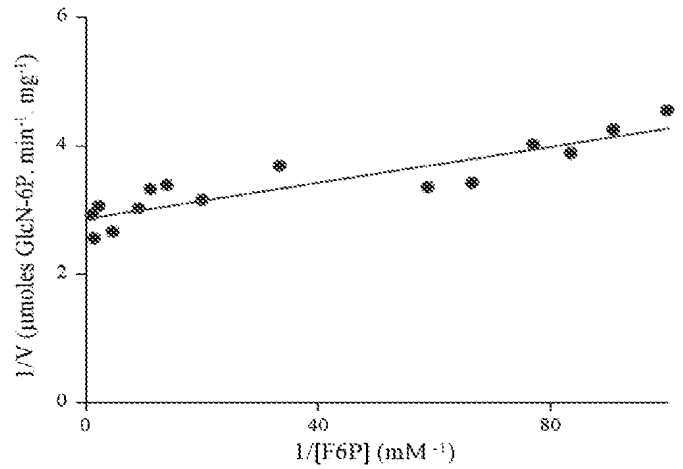

Fig. 4. Activation of rhGfat1 in glutaminase activity by Fru-6P. The activity was measured using hydrolysis of GLUPA with 3 μg rhGfat1 and 3 mM GLUPA in a total volume of 200 μL. The maximum velocity was 0.35 ± 0.01 U/mg rhGfat1. The K_d of Fru-6P was 4.9 ± 0.8 μM calculated by double reciprocal plot representation.

optimum pH of 7.5 (data not shown). The K_m of Fru-6P 0.98 mM and K_m of Gln 0.84 mM were determined by measuring the initial rate of GlcN-6P or Glu formation, respectively, with maximal velocity of 3.7 ± 0.4 U/mg and 3.2 ± 0.2 U/mg, respectively (Table 3).

Activation of the glutaminase domain by Fru-6P

The ability of Fru-6P to stimulate glutaminase activity was measured by quantifying the hydrolysis of GLUPA by the glutaminase domain of rhGfat1. Activation of glutaminase activity by Fru-6P binding to the isomerase domain exhibited saturable kinetics with a K_d of 5 μM derived from the reciprocal representation $1/V = f(1/[\text{Fru-6P}])$, a value consistent with the 2 μM K_d previously reported by Broshat et al. [15] (Fig. 4).

Discussion

L-glutamine: D-fructose-6-phosphate amidotransferase (Gfat1) which converts Fru-6P and Gln into GlcN-6P and Glu is the first and rate limiting enzyme of the hexosamine synthetic pathway (HSP) [1]. There is a strong evidence supporting a role for the HSP in the physiopathology of the complications of type II diabetes [32]. In particular, the final product UDP-GlcNAc of HSP is an activated precursor for O-linked glycosylation of proteins, responsible for tyrosine inactivation of the insulin receptor resulting in a desensitization to insulin and thus insulin resistance [33]. Therefore, rhGfat1 activity controls the level of GlcN-6P and UDP-GlcNAc, sugars implicated in type II diabetes complications. The role of hexosamine biosynthesis during the pathological development of insulin resistance highlights the need for thorough biochemical characterization of hGfat1.

As a member of the N-terminal nucleophile subfamily of amidotransferases [34] GlcN-6P synthase possesses an N-terminal cysteine that is essential for the formation of ammonia from Gln through a γ-glutamyl acyl enzyme intermediate. It was previously shown that extension of the N-terminus by a single glycine strongly affects the activity of GlmS [18]. Indeed, the Gly0 mutant version of GlmS exhibited a 40-fold decrease in affinity for Gln and an 8-fold lower glutaminase activity in terms of k_{cat} but a 5-fold decrease in affinity for Gln with 25-fold lower synthase activity in terms of k_{cat}, in comparison to its wild-type counterpart [18]. By the same token, the C-terminal part of GlmS contains crucial residues for the enzyme activity since they constitute an intramolecular channel connecting the glutaminase and isomerase domains [35]. The C-terminal, 11 amino acid-long sequence, known as a C-tail, is strongly conserved in all GlcN-6P synthases identified so far [36]. One may therefore assume that the presence of the C-terminal oligo His_6-tag should affect interdomain communication in the GlcN-6P synthase molecule and especially ammonia transfer from Gln to Fru-6P.

Chang et al. and Hu et al. [13,37] reported the expression of recombinant mammalian GlcN-6P synthases containing N-terminus-linked fusions of highly different size: glutathione-S-transferase (26 kDa) or the oligohistidyl tag (8 aa). Although the authors claimed that the fusion proteins are still active, the activity was only analyzed for the ability of the resulting enzyme to hydrolyze Gln but not for the rate of GlcN-6P formation. Olchowy et al. described three His_6-tag versions of Gfa1 from *C. albicans* and showed that the presence of either long or short His_6-tag affects GlcN-6P synthase activity especially if the tag is located at the enzyme N-terminus [38]. Expression of a fusion-Gfat therefore primarily required a clearcut analysis of the position to be tagged.

The GST-N-GlmS construct bearing the GST at the N-terminal position was a gift from Dr. J.E. Kudlow. After purification and cleavage of GST in presence of thrombine, the synthase activity was found to be 1150-fold lower than the wild type synthase activity. Based on the thrombin recognition site, the two residues left at the GlmS N-terminus (Gly and Ser) are likely to prevent catalysis thus arguing against such a construct to be used for the human Gfat. Three versions of a recombinant bacterial GlcN-6P synthase containing His_6-tag at specific positions were then constructed: the C-terminus end and positions 225 and

240 which were selected from the observation of the X-ray structure. The introduction of an internal His_6-tag (position 225) affected neither the kinetic parameters nor the structure of the bacterial enzyme, a strategy which has been successfully applied only to the ShIB protein from *Serratia marcescens* [39], RNA polymerase core sigma from *E. coli* [40] and the siderophore receptor FluA from *E. coli* [41]. Extrapolation of this information to the human enzyme resulted in the first overexpression and characterization of the internal His_6-tagged human D-glucosamine-6P synthase.

The purification of hGfat1 from various tissues has been previously shown to be very difficult because of the large number of purification steps, the low level of expression and the instability of the enzyme. The expression of recombinant enzyme with the introduction of an internal His_6-tag is then the best method to obtain an active and stable protein. RhGfat1 was obtained 95% pure in two steps with a specific activity of 3.7 U/mg. This value is 6000-fold higher than the one reported for enzyme from human liver [42], 16-fold higher than the N-terminal GST fusion protein [13] and about 9-fold higher than the enzyme described more recently by Broshat et al. [15]. Two different methods, gel filtration and equilibrium ultracentrifugation, identified human enzyme as a homotetramer as reported for rat liver enzyme [10].

The two microplate assays, respectively, based on fluorescence to quantify the glutaminase activity and on the Morgan–Elson reagent to determine synthase activity, permitted the kinetic characterization of rhGfat1. RhGfat1 exhibited K_m^{Fru-6P} and K_m^{Gln} similar (half mM range) to those found for the enzymes from *E. coli* [43], *T. thermophilus* [44], *C. albicans* [11] and rat liver [10]. The value of K_m^{Fru-6P} is in sharp contrast with the data obtained by Broshat et al. [15] who reported a K_m in the μM range (7 μM). This low value was rationalized by the sensitivity of the radiometric assay which permitted working at very low product accumulation to avoid GlcN-6P inhibition ($K_i = 6$ μM) which might have occurred with the same enzyme from all other sources. Working at very low substrate conversion could in fact afford an apparent K_m value close to the Fru-6P dissociation constant which is indeed in the micromolar range. The activation of the Gln site by Fru-6P using GLUPA as an alternate substrate allowed determination of a Fru-6P dissociation constant of 5 μM, in good agreement with the 2 μM value reported by Broshat et al. [15].

The first production of human Gfat1 with an internal His_6-tag allowing a good level of expression of an active protein should facilitate the determination of kinetic mechanisms and high throughput screening of specific inhibitors with potential applications for treatment of type 2 diabetic complications.

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